MOLECULAR STRATEGY FOR THE CONSTRUCTION OF A GENETICALLY ENGINEERED VACCINE FOR VENEZUELAN EQUINE ENCEPHALITIS VIRUS

FINAL REPORT

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Venezuelan equine encephalitis virus (VEE) full-length clones; In vitro mutagenesis of VEE full-length clones; Attentuated VEE glycoprotein mutants

Unclassified Unclassified Unclassified Unlimited

13. Abstract (continued)

mutation. Compared to TC-83, the triple mutant had lower mortality and extended survival in 1 wk old mice. In hamsters, the single and multiple mutants were inoculated ip. at two doses, 5×10^3 and 5×10^5 PFU; TC-83 was inoculated at a single dose of 5×10^4. The triple mutant was avirulent, whereas TC-83 gave 20% mortality, a value consistent with several of the single mutant recombinants. In ponies, the triple mutant was attenuated and induced solid protection to challenge with VEE-71180, a subtype IB strain which is highly virulent for equines.

FOREWORD

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Introduction

Immunization with live, attenuated vaccines has been the most effective strategy employed for the control of virus disease. As the incidence of various diseases has decreased however, the frequency and severity of complications arising from the use of live vaccines has become increasingly disturbing. These undesirable side effects result from reversion of the attenuating mutations carried by the vaccine strain, a well-known risk factor in the use of live virus vaccines. Reversion to an intermediate virulence phenotype may be involved in the adverse reactions to the restricted-use investigational vaccine for the alphavirus, Venezuelan equine encephalitis virus (VEE), an important human and veterinary pathogen. Approximately 20% of vaccinees who receive the live virus vaccine, TC-83, experience symptoms. These range from malaise to an influenza-like syndrome reminiscent of virulent VEE (Edelman, 1986).

The disadvantage of reversion to virulence could be overcome by introducing multiple, independently attenuating mutations into a candidate VEE vaccine strain. This appears to have occurred serendipitously in the case of the Sabin type 1 poliovirus vaccine which has a low reversion frequency (Omata et al., 1986). We are using an informed design process to develop analogous candidate VEE vaccine strains containing multiple attenuating mutations. A corollary of this design process is the ability to avoid unnecessary or deliterious mutations and thereby to produce the most immunogenic vaccine strain possible. The tools that we have developed for this approach are 1), a cDNA clone of the entire VEE genome placed downstream from a T7 promoter such that infectious viral RNA can be transcribed in vitro (Davis et al., 1989) and 2), a panel of rapidly-penetrating, attenuated VEE mutants isolated after a limited number of selective passages in tissue culture (Johnston and Smith, 1988). During the period covered by this report, we have accomplished most of the specific objectives of the contract.

Technical Objectives

1) To sequence a series of attenuated VEE mutants, which we had derived previously, in order to map the mutations responsible for their individual attenuation phenotypes.

2) To generate a library of complementary DNA clones representing the entire VEE genome.

3) To characterize these clones with restriction enzymes and to sequence the termini of the inserts in order to orient them with respect to each other and to the published portions of the VEE genome sequence.

4) To assemble the partial clones into a full-length VEE clone downstream from a T7 promoter which will allow *in vitro* transcription of infectious VEE RNA.

5) To introduce multiple attenuation mutations, identified in objective one, into the full-length clone either by allelic replacement or by site-directed mutagenesis.

6) To assess the effect of specific attenuation mutations, individually and in combination, on pathogenicity, immunogenicity and reversion to virulence in hamster and mouse model system.

Selection and characterization of VEE attenuated mutants

Our approach to the identification of attenuating mutations is based on our previous work with Sindbis virus. In studies of the process of attenuation by passage in cultured cells, we found that when a general selective pressure for more efficient growth was replaced with a direct selection for rapid penetration, mutants with reduced virulence were isolated after only a few selective passages. These fast-penetrating, attenuated mutants carried a single nucleotide change in a region where two functional domains of the Sindbis virus glycoprotein overlap, one domain involved in penetration of cultured cells and the other in pathogenesis.

Rapid penetration is also a selectable marker for attenuation of VEE. Several rapidly penetrating, mouse-avirulent VEE mutants were isolated after only two to four selective cell culture passages (Johnston and Smith, 1988). Table 1 shows a comparison of the E2 glycoprotein sequences of representative VEE mutants. The complete E2 gene sequence was determined for each mutant by direct sequencing of RNA genomes. For some of the mutants, the sequence of the E1, E3 and 90% of the 6K genes was determined as well.

All but two of the mutants showed a single nucleotide change in the E2 gene that altered one of four codons, 76, 81, 120, or 209 (numbered from the amino terminus). E2 position

120 is the site of one of the seven amino acid changes between the virulent VEE Trinidad donkey strain and the TC-83 vaccine strain, which has an arginine at this position (Johnson et al., 1986). All of the amino acid substitutions involve significant charge changes; however, only the change from ser to lys at position 120 would cause a change in the hydropathic profile of the E2 polypeptide. Amino acids 76 and 120 are predicted to occur in slightly hydrophobic contexts, while residue 209 is found in a hydrophilic region near both a proposed N-linked glycosylation site and a region predicted to have antigenic properties. This prediction was verified by the observation of Kinney et al. (1988) that expression of a recombinant vaccinia virus containing a VEE E2 gene with a substitution of lys for glu at E2 209 greatly reduced detection of the $E2^h$ epitope in cells infected with the recombinant virus. In addition, a neutralization escape mutant selected with an $E2^h$-specific monoclonal antibody contained a mutation at E2 residue 207 (Johnson et al., 1990). These observations indicate that the change from a glu to a lys at E2 209 significantly altered an epitope recognized by a neutralizing monoclonal antibody. However, as shown below, a molecularly cloned strain which carries this mutation retains the ability to elicit protective immunity against a high dose challenge with virulent virus.

Three of the VEE mutants derived from the rapid penetration selection share an E2 mutation (E2 gln 81 to E2 arg 81) but differ phenotypically. All three are rapidly penetrating but one is virulent and two are attenuated. We have not yet completed the sequencing of the E2 and E1 genes of these mutants, but we anticipate one of two possible explanations of this paradox. The E2 arg 81 mut4444ation may confer a rapid penetration phenotype without changing virulence, in which case the two attenuated mutants should contain an additional attenuating mutation(s) elsewhere. Alternatively, the E2 arg 81 mutation may confer both rapid penetration and attenuation, and the virulent mutant would contain a second-site suppressor mutation which causes phenotypic reversion to virulence without affecting rapid penetration. The Sindbis model offers an example of the latter situation (Pence et al., 1990). We are currently introducing the E2 arg 81 mutation into the full-length clone in order to distinguish these alternatives.

Two double mutants also were found. One of these contained a mutation at position 245 in addition to the change at 209. The other showed two changes at positions 3 and 4 of E2,

from glu-3, glu-4 to lys-3, lys-4. The effect of this double mutation on the maturation cleavage between E3 and E2 is of interest in light of previous work with an attenuated mutant of the S.A.AR86 strain of Sindbis. The S.A.AR86 mutant fails to cleave E3 from E2 due to an extra glycosylation site at position 1 of E2 (Russell et al., 1989). In addition to being attenuated in mice, this cleavage-defective mutant grows extremely slowly in mosquito cells (C6/36) and is much less likely to be disseminated in intact mosquitoes infected orally in artificial feeding experiments (Presley et al., 1991).

Construction of a VEE cDNA clone

The library of cDNA clones produced from purified virion RNA of the virulent Trinidad donkey strain of VEE (VEE-TRD) and the procedures used in construction of clone pV1000 have been presented in detail elsewhere (Davis et al., 1989). Briefly, cDNA clones were derived by the method of Gubler and Hoffman (1983) and mapped by extensive restriction analysis (Figure 1). Two clones, which together constituted approximately the 5' half of the VEE genome, were combined. Site-directed mutagenesis was used to insert a modified T7 RNA polymerase promoter and the first 8 nucleotides of the VEE sequence upstream of the 5'-most primary clone. A 3' half clone also was assembled from two primary clones, and a unique Not I site was inserted downstream from the poly (A). As shown in Figure 2, the two half clones were combined to form clone pV1000. The completed clone includes, in the following order, approximately 1.8 kb of pBR322-derived vector sequences from the full-length Sindbis clone, Toto1101 (Rice et al., 1987), the untranscribed portion of the consensus T7 promoter, a single G residue, a sequence of approximately 11 kb predicted to represent the entire VEE genome, a 21-nucleotide poly (A) tract and a unique Not I restriction site. Clone pV1000 DNA was linearized by digestion with Not I and incubated with T7 RNA polymerase in a standard transcription reaction. An RNA with the electrophoretic mobility of VEE virion RNA was synthesized. When this reaction mixture was used to transfect DEAE dextran-treated chick embryo fibroblasts, a typical VEE cytopathic effect was observed. Culture supernatants from the transfected chick cells contained over 10^7 PFU when assayed on vero cells, and when we transferred the supernatants to fresh uninfected BHK cells, CPE appeared within 16 hrs. The infectivity of the transcription reaction was sensitive to RNase, but not DNase, except when the DNase was added before transcription. The transfected chick cells

also contained VEE antigens as shown by reaction of acetone-fixed cells with VEE-specific polyclonal antibodies and an anti-VEE monoclonal antibody (provided by John Roehrig). These results strongly suggested that pV1000 was the template for transcription of infectious VEE RNA.

Further study of pV1000 revealed that it carried a 102-nucleotide in-frame deletion at the 3'-end of the nsP3 gene that originated when the 5' and 3' half clones were joined in the final step of the construction. The deletion was present in the genomes of virions recovered from transfected cells, providing formal proof that the cloned VEE genome sequence was the origin of the progeny virus. The deletion occurred in a region containing two somewhat divergent 102-nucleotide repeat units in tandem. The deletion mutant retained a single copy of the repeat as well as a considerable portion of a large predicted secondary structure formed by the two repeats. The deleted nsP3 sequence does not appear to be necessary for growth in chick, vero or BHK cells, or for virulence in mice. Studies are proceeding to determine the role of this nsP3 sequence in the VEE life cycle.

The complete genome sequence was restored in the plasmid, pV2000, using a modification of the procedure which produced the deleted plasmid, pV1000. In the modified procedure, a preparation of plasmid pT7 5'V50 (Figure 2) was partially digested with Xho I and then completely digested with Xba I to produce a heterogeneous population of DNA fragments, some of which contained the 102-nucleotide region deleted from pV1000. Following ligation with the appropriate Xba I-Xho I fragment from pTX 3'V50 (Figure 2), constructs with the complete VEE sequence, including pV2000 were isolated.

Insertion of attenuating mutations into the VEE cDNA clone by site-directed mutagenesis

The structural gene region of the repaired full-length VEE clone pV2000 was subcloned into M13 and used as the substrate for *in vitro* mutagenesis (Kunkel, 1985). A noncoding change which eliminated a SmaI site was introduced into the subclone so that sequences derived from the subcloned genes could be distinguished from analogous sequences in pV2000 by restriction analysis. In this background, individual subclones were produced, each of which contained a candidate attenuating mutation, and the mutated E2 genes were substituted separately for the wild-type VEE

E2 gene of the full-length clone, pV2000. These constructs represent a series of isogenic VEE cDNA clones that differ by a single nucleotide. A control construct, designated pV2000/SmaI, contained an unmutated E2 gene (except for the noncoding change in the SmaI site) derived from the M13 subclone. Multiple mutants were produced by applying the mutagenesis procedure to a clone already containing mutations at one or more of the other loci. The constructs are indicated in Table 2.

Evaluation of mutant recombinants in animal models

Preliminary evaluation of mutant recombinant VEE strains (Table 2) has been initiated. All of these proved avirulent in adult mice inoculated with 10^4 PFU, ip. The positive control for this experiment consisted of virus derived from the unmutated clone pV2000/SmaI lacking only the diagnostic SmaI site. This virus was uniformly lethal and was indistinguishable from TRD. The negative control consisted of an equivalent dilution of medium from a BHK culture transfected with untranscribed, linearized pV2000. Of the 60 mice inoculated with the mutant recombinants, 56 resisted challenge with 10^4 TRD, ip., which is generally consistent with prechallenge ELISA and FA evaluation of their sera. All 10 mice which received the triple mutant were resistant to challenge.

The recombinants also were inoculated into younger mice (8-9 day or 15-16 day) to discern differences in the degree of attenuation specified by their constituent mutations (Table 2). While all of the mutant constructs exhibited reduced virulence as indicated by extended survival, the single mutants tested to date could be ranked in order of increasing attenuation, from E2 lys 120 to E2 lys 209 to E2 lys 76 which was the most attenuated. Stated another way, mice exhibit an age-related resistance to the mutants with each mutant having a characteristic age at which mice become resistant to it. Just as E2 lys 76 seemed to be the most strongly attenuating single mutation, the double mutants having E2 lys 76 as one of the mutations (76/120 and 76/209) were more attenuated than the 120/209 double mutant. The triple mutant was more attenuated than any of the double mutants. Moreover, mice 8-9 days of age which were infected with the triple mutant had an extended average survival time compared to those inoculated with TC-83.

We have also examined virulence in different adult animal models. Hamsters die during the initial lymphotropic/myelotropic stage of VEE infection, prior to the point where central nervous system involvement is clinically apparent. Virulence for these animals, therefore, is a sensitive test of the ability to carry out this first stage of pathogenesis. The individual mutations differ in their virulence for hamsters, with the mutation at E2 position 76 being the most attenuated (Table 2). This observation is consistent with the hypothesis that the defect caused by the mutation at E2 residue 76 may be expressed during the early lymphotropic stage of the disease. In hamsters, the triple mutant was avirulent at ip. doses 3 to 5 orders of magnitude higher than the VEE-TRD LD_{50}, while similar doses of TC-83 gave 20% mortality.

Our results to date suggest 1) that like the Sindbis model, attenuating loci in VEE differ with respect to the degree of attenuation, 2) that mutant combinations are generally more attenuated than constructs having the constituent single mutations, and 3) that in these rodent models, our initial recombinant triple mutant already compares favorably with TC-83.

The TC-83 E2 gene differs from TRD-VEE at 5 loci, one of which is at position 120 where TC-83 has a thr to arg mutation (Johnson et al., 1986). Our data showed that TC-83 was considerably more attenuated in hamsters and 1 wk old mice than the single mutant construct having E2 lys 120. O..e explanation for this difference in attenuation is that the lys substitution in the construct may not be as effective in attenuating VEE as an arg substitution as found in TC-83. However, as lys and arg are both large basic amino acid residues, the more likely explanation may be that one or more of the other coding differences between TC-83 and TRD-VEE contribute significantly to its attenuation in these rodent models. In the hamster model, TC-83 had 20% mortality, a result similar to several of the constructs harboring single-site mutations. This is consistent with the suggestion that attenuation of TC-83 for hamsters, and the reduced lymphotropism that hamster attenuation implies, may reside in a single TC-83 locus. *In vivo* reversion at this single locus may account for some of the adverse reactions encountered in human TC-83 vaccinees.

The triple mutant and each of the individual E2 mutations at 76, 120 and 209 have been inoculated into ponies in collaboration with the USDA-ARS facility at Plum Island, New York. Temperature charts for individual animals inoculated with PBS, the unmutated clone pV2000/SmaI, TC-83, and the triple mutant candidate are shown in Figure 3a-d. Although evaluation of the data from this experiment is not yet complete, several preliminary conclusions may be drawn. 1) The triple mutant was essentially avirulent in ponies following inoculation of 5 x 10^4 PFU, sc.. Four of five animals showed no overt clinical signs and the fifth had an elevated temperature for one day. 2) A higher proportion of the animals receiving the single mutants exhibited symptoms, but these were delayed and much less severe than observed in ponies inoculated with control virus derived from pV2000/SmaI, the unmutated, virulent clone. 3) All of the vaccinated animals were solidly protected against challenge (10^4 PFU, sc.) with VEE-71180, a subtype IC strain highly virulent for equines.

Progress Summary

At the time of this submission, our progress may be summarized as follows. We have sequenced the glycoprotein genes of 11 rapidly penetrating, attenuated mutants isolated previously; sequencing of the remaining two mutants is in progress. Five putative attenuating loci have been identified. We have constructed a full-length cDNA clone of TRD-VEE which, when transcribed *in vitro*, yields an infectious RNA. VEE virus derived from the clone is indistinguishable from TRD-VEE in cell culture and *in vivo*. Full-length clones containing each of the putative attenuating mutations individually have been constructed using site-directed mutagenesis. Using virus derived from these constructs, we have reproduced the attenuated phenotypes of the biological mutants. Similarly, we have combined the individual attenuating mutations to derive double and triple mutant clones. Virus produced from the single, double and triple mutant constructs tested to date have been avirulent in adult mice (at 10^4 PFU, ip.) and induced protective immunity to challenge with 10^3 PFU of TRD-VEE. Experiments in 1 wk old mice allowed the discrimination of degrees of attenuation among the constructs and showed that the multiple mutants were more attenuated than those containing a single attenuating mutation. Compared to TC-83, the triple mutant had lower mortality and extended survival in 1 wk old mice. In hamsters, the single and multiple mutants were inoculated ip. at two doses, 5 x 10^3 and 5 x 10^5 PFU; TC-83

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Figure Legends

Figure 1. VEE cDNA clone library and partial restriction map. The alignment of selected cDNA clones was based on results of restriction enzyme digests and sequence analysis. Sites for 19 enzymes are shown. Map distances in the nonstructural gene region were estimated from relative migration rates of restriction fragments in agarose gels. Vertical bars show some of the restriction sites mapped on the cDNAs. Structural genes are designated C (capsid), E3, E2, 6K, and E1. V11, V12, and V13 are oligonucleotide sequencing primers. $(A)_n$, poly(A) tract; A, Afl II; B, BamH I; Bs, BssH II; E, EcoR I; H, Hind III; K, Kpn I; N, Nae I; Nd, Nde I; P, Pst I; S, Sac I; Sa, Sal I; Sc, Sac II; Sh, Sph I; Sm, Sma I; Sp, Spe I; St, Stu I; T, Tth111 I; X, Xba I; Xh, Xho I.

Figure 2. Scheme for construction of a candidate full-length cDNA clone of VEE downstream from a T7 promoter. The T7 promoter (→) is adjacent to the VEE genome 5'-end. The $(A)_{21}$ tract in clone pTX 67-12(N) is adjacent to the unique Not I site. Boldface arcs describe viral cDNA inserts. The approximate positions of restriction sites are marked.

Table 1

Candidate Attenuating Mutations in the E2 Glycoprotein Genes of Rapidly Penetrating, Mouse Avirulent Mutants of VEE

	E2 amino acid residue						
	3	4	76	81	120	209	245
Virus Strain[a]							
VEE-TRD	glu	glu	glu	gln	thr	glu	lys
FD-3-6	LYS	LYS	glu	gln	thr	glu	lys
FD-3-9b[b]	glu	glu	glu	ARG	thr	glu	lys
FD-3-13[b]	glu	glu	glu	ARG	thr	glu	lys
FC-4-2	glu	glu	LYS	gln	thr	glu	lys
FC-4-7	glu	glu	glu	gln	LYS	glu	lys
FC-1-2	glu	glu	glu	gln	thr	LYS	lys
FC-4-6	glu	glu	glu	gln	thr	LYS	ASN

[a]VEE-TRD is the virulent parent strain from which the mutants were derived. The mutant designations identify either the Fort Detrick strain (FD) or the Fort Collins strain (FC) of VEE-TRD as the parent, and indicate the passage number followed by the number of the isolate. E2 amino acid residues are numbered from the amino terminal end of the mature protein.

[b]A third rapidly penetrating mutant that retained the parental phenotype of virulence in mice, FD-2-2, was also found to carry this mutation.

FIGURE 1

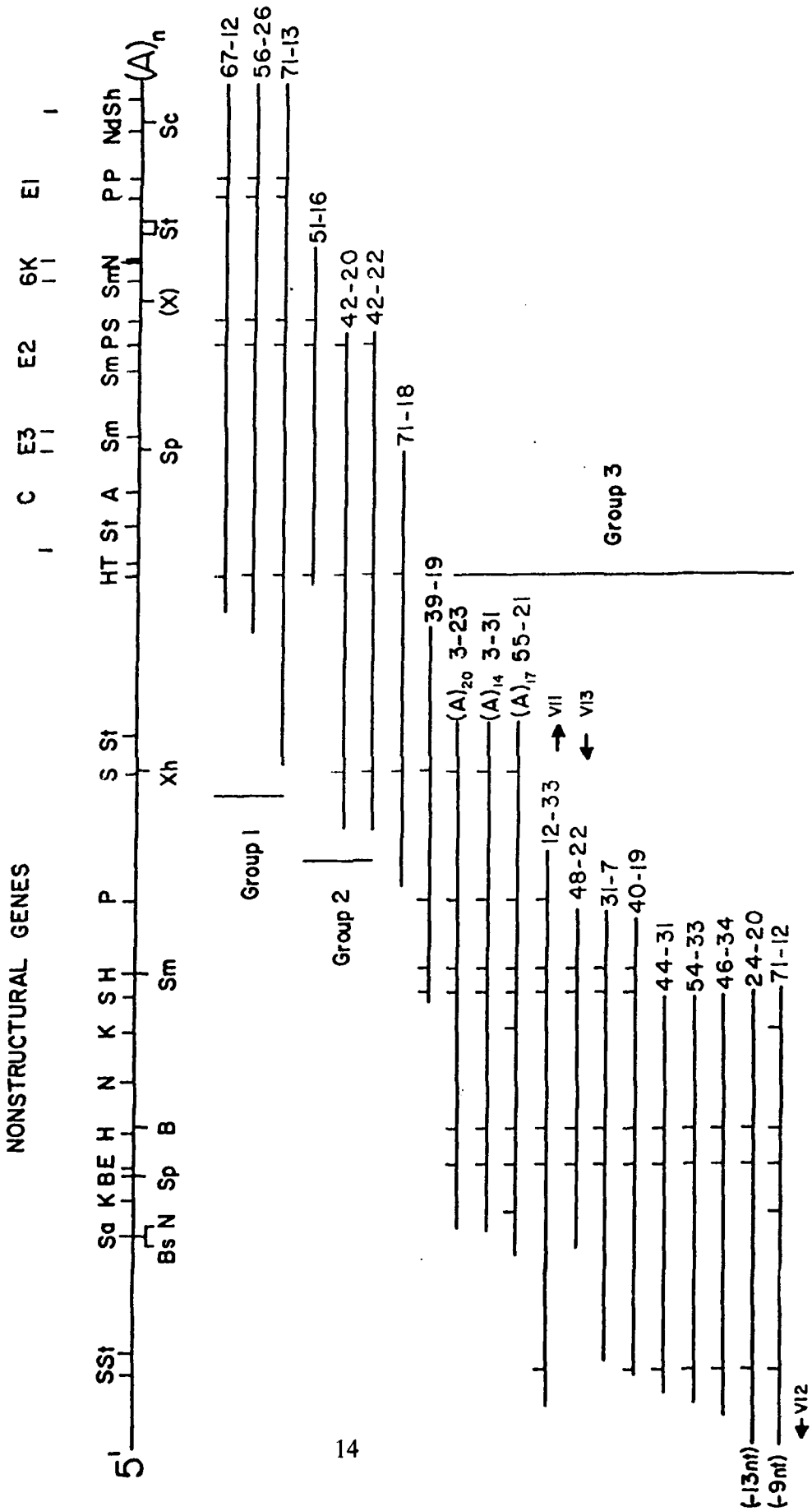
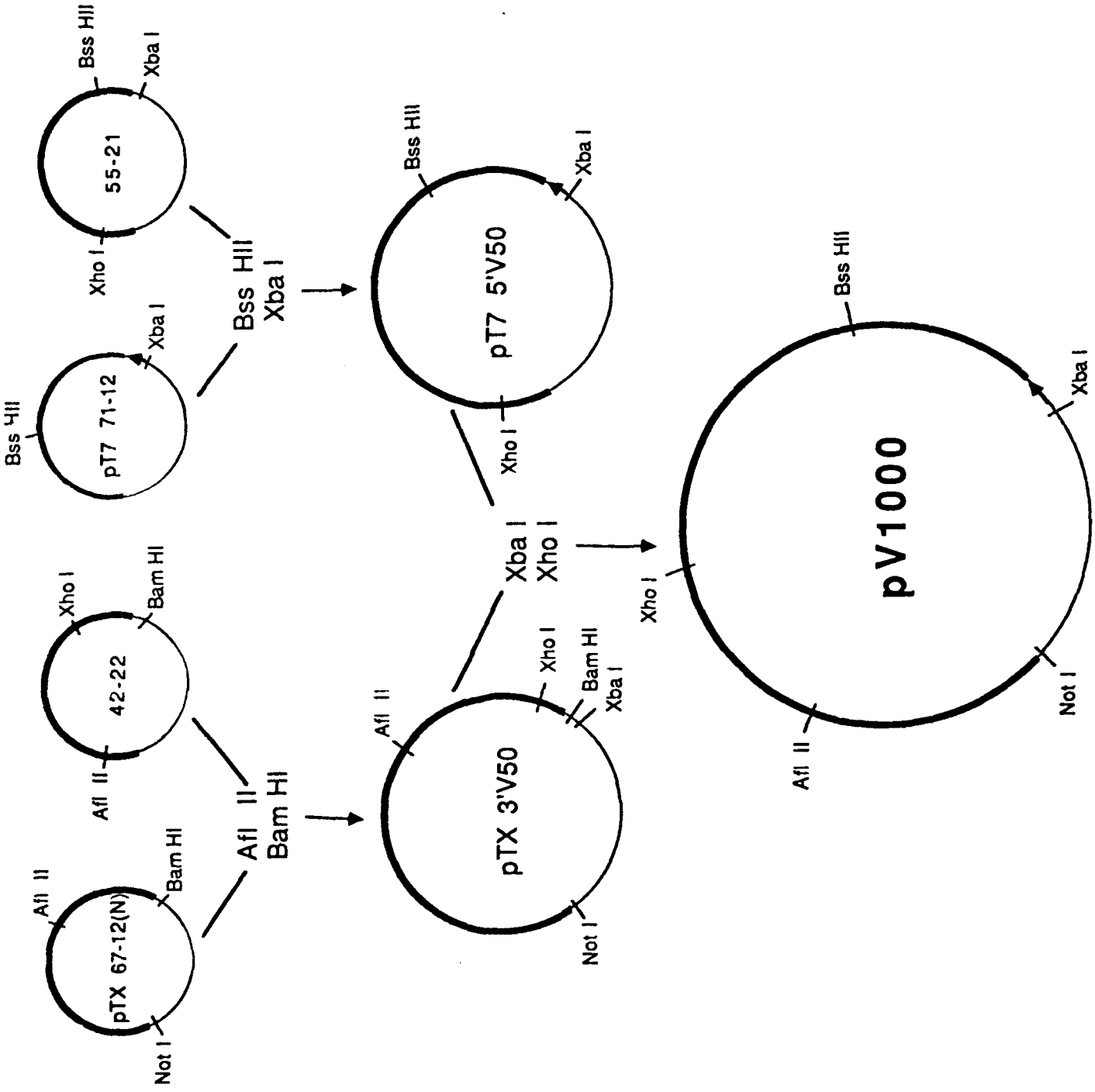

NONSTRUCTURAL GENES
5'
SSt
Sa K B E H N K S H P S St
Bs N Sp B Sm Xh
I C E3 E2 6K E1 I
HT St A Sm Sm PS SmN PP NdSh
Sp (X) St Sc
(A)n
67-12
56-26
71-13
51-16
42-20
42-22
71-18
39-19
(A)20 3-23
(A)14 3-31
(A)17 55-21
12-33
48-22
31-7
40-19
44-31
54-33
46-34
24-20
71-12
Group 1
Group 2
Group 3
VII
V13
V12
(-13nt)
(-9nt)

FIGURE 2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Afl II
Bam HI
Not I
42-22
Xho I
Bam HI
Afl II
pT7 71-12
Bss HII
Xba I
55-21
Bss HII
Xba I
Xho I
Afl II
Bam HI
Bss HII
Xba I
pTX 3'V50
Afl II
Xho I
Bam HI
Xba I
Not I
Xba I
Xho I
pT7 5'V50
Bss HII
Xba I
Xho I
Xho I
pV1000
Bss HII
Xba I
Not I
Afl II

Table 2

Site-Directed Mutants of Venezuelan Equine Encephalitis Virus

	Percent Mortality (Average Survival Time)			
	9 Day Mice[a]	15 Day Mice[a]	Adult Mice[b]	Adult Hamsters[c]
RP Mutants[d]:				
E2 3/4, E→K/E→K			0	60 (4.8)
E2 76, E→K	82 (8.8)	9 (9.0)	0	30 (5.3)
E2 120, T→K	100 (5.6)	100 (6.9)	0	100 (4.5)
E2 209, E→K	100 (5.5)	40 (7.8)	0	60 (5.2)
Double Mutants:				
E2 76 & 120	50 (7.2)	30 (9.3)	0	
E2 120 & 209	100 (6.8)	40 (10.8)	0	
E2 76 & 209	64 (8.9)	0	0	
Triple Mutant:				
E2 76, 120 & 209	45 (10.2)	0	0	0
Controls:				
TC-83	55 (8.8)	0	0	20 (6.0)
pV2000/SmaI[e]	100 (3.0)	100 (5.3)	100 (10.8)	100 (3.1)

[a]ICR mice inoculated with 5 x 10^3 PFU ip.

[b]C57Bl/6J inoculated with 1 x 10^4 PFU ip.

[c]5 x 10^5 PFU inoculated ip., except for TC-83 (5 x 10^4 PFU).

[d]Mutations identified by sequencing attenuated, rapid penetration mutants isolated by Johnston and Smith (1988).

[e]This construct contains a single non-coding change which ablates a SmaI site.

Figure 3

Temperature Status of Ponies Inoculated with VEE

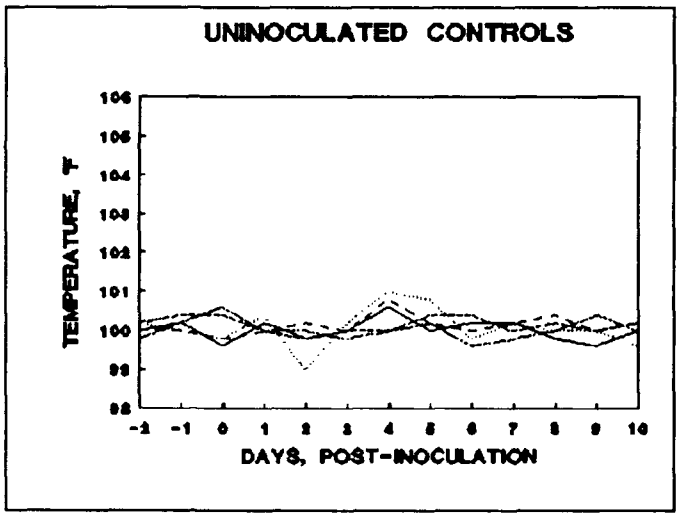

Figure 1a

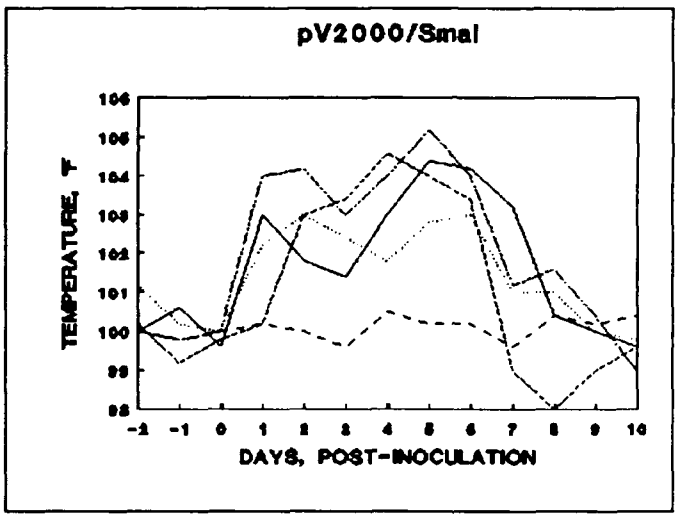

Figure 1b

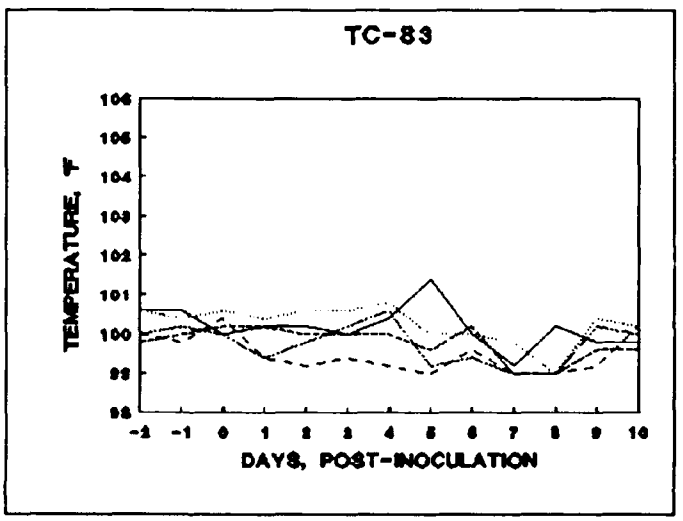

Figure 1c

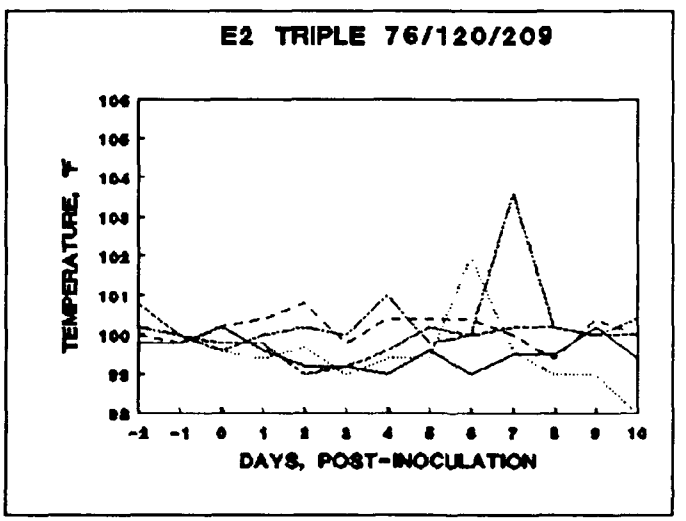

Figure 1d

Groups of ponies were inoculated with either PBS (Panel 1a), virus from the unmutated parent clone, pV2000/SmaI (Panel 1b), TC-83 (Panel 1c) or the triple mutant candidate (Panel 1d) at 5 x 10^4 PFU sc. Rectal temperatures were recorded from each animal daily; each line represents the temperatures recorded from an individual animal.

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